Exhibit A

Exhibit A

Priority paper

Transcriptional activation by amphipathic carboxylic peroxisomal proliferators is induced by the free acid rather than the acyl-CoA derivative

Rachel HERTZ, Ina BERMAN and Jacob BAR-TANA

Department of Human Nutrition and Metabolism, Faculty of Medicine, Hebrew University, Israel

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Most peroxisomal proliferators consist of a carboxylic group attached to a hydrophobic backbone yielding an amphipathic carboxylate molecule. The respective CoA derivatives of peroxisomal proliferators, formed by ATP-dependent CoA thioesterification catalyzed by long-chain-acyl-CoA synthase, have been repeatedly considered as the immediate inducers of peroxisome and other genes. In this study, the putative requirement for prior CoA thioesterification of peroxisomal proliferators was evaluated by analyzing the induced expression of a reporter plasmid promoted by the peroxisomal acyl-CoA-oxidase promoter in cells transiently cotransfected with expression vectors for the peroxisome-proliferator-activated receptor and the long-chain-acyl-CoA synthase. Transcriptional activation of peroxisomal acyl-CoA oxidase by peroxisomal proliferators was inhibited in the presence of transfected functional acyl-CoA synthase. The inhibitory effect was negatively correlated with the capacity of the acyl CoA synthase to catalyze CoA thioesterification of the respective proliferator. Hence, the immediate inducer is the peroxisomal proliferator free acid rather than the respective CoA derivative or a metabolite derived from the peroxisomal-proliferator-CoA intermediate

The number of liver peroxisomes may be markedly increased in rodents and some other species by native long-chain fatty acids or xenobiotic aryloxyalkanoic fibrates, substituted long-chain dicarboxylic acids, phthalate or adipate plasticizers and other effectors collectively defined as peroxisome proliferators [1, 2]. The increase in peroxisomes is accompanied by a differential induction of specific peroxisomal [e.g. acyl-CoA oxidase (AOX)] and non-peroxisomal (e.g. cytochrome *P*450 IV A6) proteins accounted for by peroxisomal-proliferator-induced transcriptional activation of the respective genes [3]. Transcriptional activation exerted by peroxisome proliferators has been recently found to be mediated by a new member of the steroid/thyroid hormone receptor superfamily, namely the peroxisome-proliferator-activated receptor (PPAR) [4, 5]. PPAR may *trans*-activate transcription by binding to sequence specific peroxisomal-proliferator-response elements (PPRE) in the 5′-flanking promoters of the concerned genes [6–8]. However, the putative binding of peroxisome proliferators to PPAR still remains unclear [4].

Correspondence to J. Bar-Tana, Department of Human Nutrition and Metabolism, Faculty of Medicine, The Hebrew University, P O Box 12272, IL-91120 Jerusalem, Israel

Abbreviations. AOX, peroxisomal acyl-CoA oxidase; PPAR, peroxisome-proliferator-activated receptor; PPRE, peroxisome-proliferator-response element; Wy-14,643, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]-acetic acid; nafenopin, 2-methyl-2-[*p*(1,2,3,4-tetrahydro-1-naphthyl)-phenoxy]-propionic acid; bezafibrate, 2-[4-(2-[4-chlorobenzamido]-ethyl)phenoxy]-2 methyl-propanoic acid.

Enzyme. Long-chain-acyl-CoA synthase (EC 6.2.1.3).

In spite of their apparent structural heterogeneity [1, 2], peroxisome proliferators may be characterized by a common structural feature [9]. Indeed most peroxisome proliferators consist of a carboxylic group attached to a hydrophobic backbone yielding an amphipathic carboxylate molecule. The carboxylic function may either be present initially [e.g. in most fibrate drugs, β,β'-tetramethylhexadecanedioic acid, and native long-chain fatty acids] or may be derived by metabolic conversion of the respective prodrugs (e.g. tiadenol and phthalate plasticizers) into the active proliferator (e.g. dicarboxylic thia acid and 2-ethylhexanoic acid). The free carboxylic acid or a metabolite thereof may thus be directly involved in the inductive process [9].

Peroxisome-proliferator-CoA derivatives, rather than the respective free acids, have indeed been repeatedly considered as the immediate inducers of peroxisomes and the relevant genes. These suggestions were first initiated due to the reported peroxisome-proliferative capacity of native long-chain fatty acids [9, 10] taken together with the central role played by long-chain fatty-acyl-CoAs rather than the respective free acids in fatty-acid metabolism. Furthermore, recent observations concerning the substrate availability of xenobiotic amphipathic carboxylates for the long-chain-acyl-CoA synthase [11–14], the induction of the long-chain-acyl-CoA synthase by peroxisome proliferators [15], and the role played by the formed CoA derivatives in modulating protein-kinase C activity [16], in forming xenobiotic mixed lipids [17] or xenobiotic acylated proteins [18], apparently corroborated the putative role suggested for peroxisome-proliferator-CoA, or

a derivative thereof, in inducing transcriptional *trans*-activation of peroxisome and other genes. Lack of evidence for direct binding of free peroxisome proliferator to PPAR [4], in contrast to the general paradigm for other members of the steroid/thyroid hormone receptor superfamily, could be further considered as an indication of the putative ligand role of peroxisome-proliferator metabolites rather than the respective free acid.

In this study, the putative requirement for prior CoA thioesterification of proliferators in the course of proliferator-mediated transcriptional activation of peroxisomal genes was evaluated by analyzing chloramphenicol acetyltransferase expression induced by added proliferator in cells transfected with an expression vector for the long-chain-acyl-CoA synthase together with an expression vector for PPAR and a reporter-gene construct consisting of the AOX promoter linked to chloramphenicol acetyltransferase.

MATERIALS AND METHODS

Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco). For transfection, cells were plated onto 60-mm-diameter dishes and transfected with CsCl-purified plasmid DNAs added by calcium phosphate precipitation. The β-galactosidase expression plasmid *pRSGAL* (2 μg) added to each precipitate served as an internal control for transfection. Cells were transfected for 5 h, washed and treated with ligands added as 1000× stock solutions in dimethyl sulfoxide for 42 h. Cell extracts were prepared by freeze/thawing and were assayed for β-galactosidase and chloramphenicol-acetyltransferase activities as described [19]. Chloramphenicol acetyltransferase activities (v) for individual cultures were expressed relative to β-galactosidase activities obtained for the same culture. The reciprocal velocities ($1/v$) were plotted against the reciprocal ligand concentrations ($1/[S]$). In all cases, a reasonable linear relationship was obtained and data points were fitted to the appropriate kinetic equation as previously described [20].

Plasmid constructs

An expression vector for the long-chain-acyl-CoA synthase was constructed by subcloning into the Bluescript plasmid the *Eco*RI–*Pst*I 2.2-kb segment of pRACS/5 [21] (kindly donated by T. Yamamoto, Japan) containing the coding region for the long-chain-acyl-CoA synthase. Following digestion of the Bluescript plasmid with *Sma*I and *Cla*I, the gel-purified 2.2-kb segment was inserted into the *CMV4* mammalian expression vector [22].

The *pSG5-PPAR* expression vector [4] was kindly donated by S. Green, England. The reporter vector for peroxisomal AOX containing the 1.3-kb Δ-472/−129 5′-flanking sequence of the *AOX* gene linked to chloramphenicol acetyltransferase (*AOX PPRE-CAT*) [6] was kindly donated by T. Osumi, Japan.

Acyl-CoA-synthase activity

The ability of the long-chain-acyl-CoA synthase expression vector transfected into COS-7 cells to direct the expression of long-chain-acyl-CoA synthase was confirmed by measuring the ATP/CoA-dependent formation of [1-^{14}C]-palmitoyl-CoA [15] in freeze/thawed extracts of COS-7 cells, as compared with extracts derived from COS-7 cells transfected with the *CMV4* vehicle vector only. Long-chain acyl-CoA synthase expression in COS-7 cells resulted in a 2–3-fold overall increase in [1-^{14}C]palmitoyl-CoA formation. It is worth noting, however, that the total increase in acyl-CoA-synthase activity in successfully transfected cells is much higher than 2–3-fold since the acyl-CoA activity is an average value for all cells in culture, whereas only a limited number of cells are successfully transfected.

ATP-dependent CoA thioesterification of peroxisome proliferator to the respective CoA derivative, catalyzed *in vitro* by rat liver microsomal long-chain-acyl-CoA synthase, was evaluated using butanol-extracted freeze-dried rat liver microsomes [23]. The activity was measured by using the respective radioactively labelled acid [15] or by following the incorporation of [^{3}H]CoA (NEN) into the acyl-[^{3}H]CoA product as described [24].

RESULTS AND DISCUSSION

The putative requirement for ATP-dependent CoA thioesterification of peroxisome proliferators for induction of the peroxisomal *AOX* gene was evaluated by following the effect of overexpressing the long-chain-acyl-CoA synthase in a cell system engaged in PPAR-mediated transcription of a reporter gene consisting of the *AOX* promoter linked to chloramphenicol acetyltransferase (*AOX PPRE-CAT*). If the immediate peroxisome-proliferator inducer of *AOX* is the respective acyl-CoA or a derivative thereof rather than the respective free acid, overexpressing the long-chain acyl-CoA synthase could be expected to increase the inductive efficacy of the proliferator as a function of its respective availability as substrate for the acyl-CoA synthase. However, if the immediate inducer of *AOX* is the proliferator free acid, or a non-acyl-CoA metabolite directly derived from the free acid, overexpressing the long-chain-acyl-CoA synthase could be expected to decrease the inductive efficacy of peroxisome proliferators serving as substrates for the long-chain-acyl-CoA synthase. Hence, in this study the effect of overexpressing the acyl-CoA synthase on the inductive efficacy of peroxisome proliferators was evaluated using a variety of proliferators which are either readily available (e.g. arachidonic acid and β,β'-tetramethylhexadecanedioic acid) or poorly available (e.g. fibrate drugs), as substrates for the acyl-CoA synthase.

As shown in Fig. 1A and in agreement with previous studies [10], PPAR-mediated transcriptional *trans*-activation of the reporter *AOX PPRE-CAT* construct in cells lacking the transfected acyl-CoA synthase was induced by arachidonic acid added to the culture medium with an apparent K_m value for arachidonic acid of 11.5 μM. The K_m value for arachidonic acid as an inducer of the reporter construct was however increased approximately eightfold in cells cotransfected with the acyl-CoA synthase. The inhibition exerted by the transfected synthase was accounted for by its function in catalyzing acyl-CoA formation as verified by transfecting the cells with a non-productive expression vector carrying the acyl-CoA synthase gene in the opposite orientation. The K_m for arachidonic acid under the non-productive conditions did not differ from that observed in cells lacking the transfected synthase altogether (Fig. 1A). The competitive pattern observed (Fig. 1A) further indicates that inhibition of arachidonic-acid-induced *AOX* transcriptional *trans*-activation by

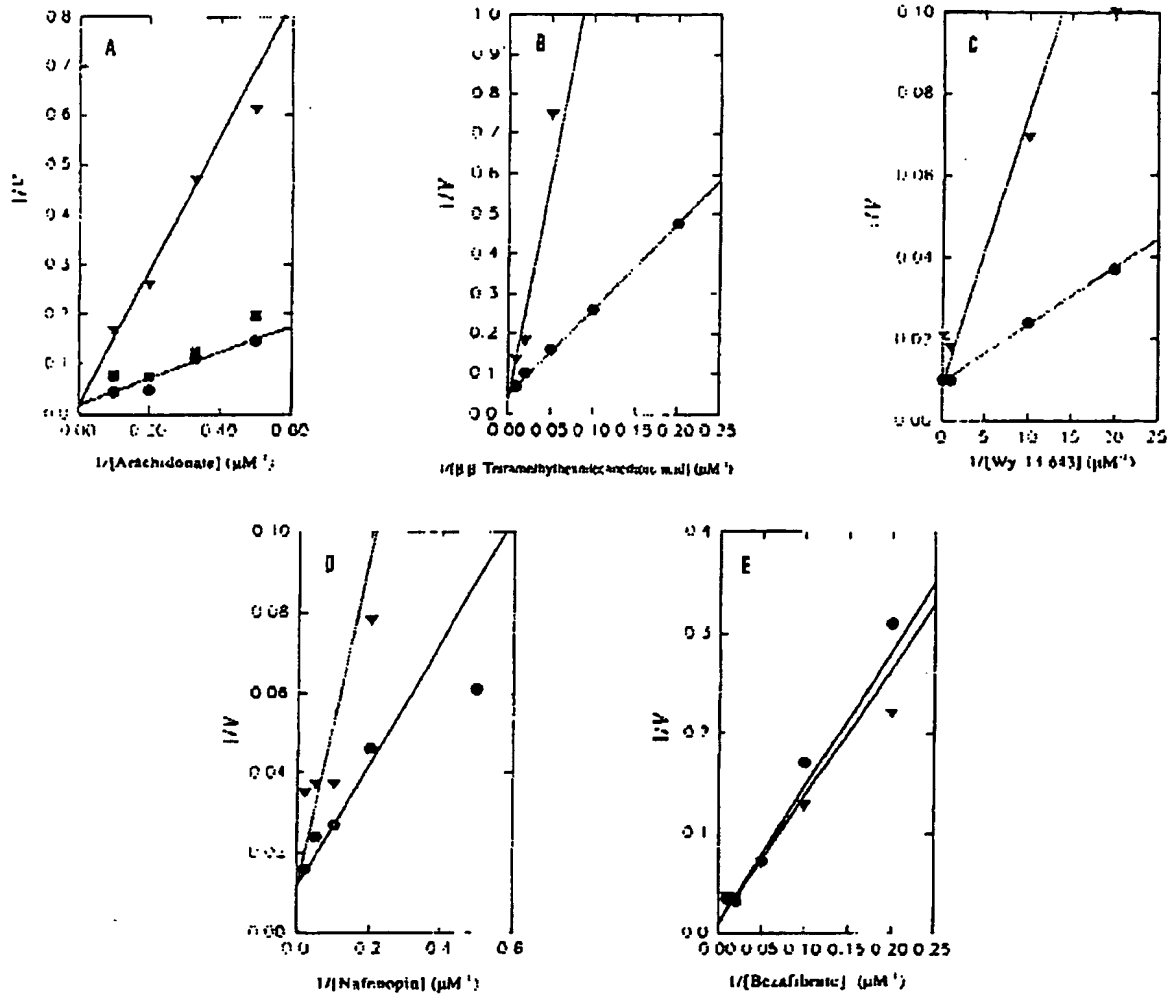

Fig. 1. Inhibition of PPAR-mediated induction of *AOX PPRE-CAT* by transfected acyl-CoA synthase. COS-7 cells were transfected with the *AOX PPRE CAT* reporter plasmid and the *pSG5 PPAR* and *pRSGAl* β-galactosidase expression vectors as described in the Materials and Methods section, in the absence (●) or presence of either a functional (▼) or a non-productive (■) expression vector for the long chain acyl-CoA synthase. Following transfection, cells were incubated in the presence of varying concentrations of arachidonic acid (A), β,β′-tetramethylhexadecanedioic acid (B), Wy-14,643 (C), nafenopin (D), or bezafibrate (E) as indicated. The chloramphenicol-acetyltransferase activity (% chloramphenicol acetylation) for individual cultures was determined as described in the Materials and Methods section and was expressed relative to β-galactosidase activity ($1.0\ A_{420}$) obtained for the same culture (V). The reciprocal velocities ($1/v$) are plotted against the reciprocal ligand concentrations. The data points of reciprocal plots were fitted to a competitive pattern as described in the Materials and Methods section. One representative experiment out of three (for arachidonic acid, β,β′-tetramethylhexadecanedioic acid and bezafibrate) and one experiment out of two (Wy-14,643 and nafenopin) are shown.

overexpressing the acyl-CoA synthase may be compromised by saturating the system with the free arachidonic acid. Hence, the lower efficacy of arachidonic acid in inducing *AOX* transcriptional activation in cells enriched with a functional acyl-CoA synthase is due to decreased availability of the free acid rather than inhibition exerted by the arachidonic-acid-CoA product.

A similar competitive inhibitory effect of the transfected acyl-CoA synthase was observed in the case of β,β′-tetramethylhexadecanedioic acid (Fig. 1B) and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]-acetic acid (Wy-14,643; Fig. 1C). The K_m values for β,β′-tetramethylhexadecanedioic acid and Wy-14,643 as peroxisome proliferators were increased approximately 4–6-fold in cells overexpressing the acyl-CoA synthase.

The inhibitory effect of the transfected acyl-CoA synthase was, however, less pronounced upon inducing the PPAR-mediated transcriptional *trans*-activation of the *AOX PPRE-CAT* construct by fibrate drugs which serve as poor substrates for the long-chain-acyl-CoA synthase [11]. Thus, overexpressing the long-chain-acyl-CoA synthase to an extent resulting in 6–8-fold inhibition of the inductive efficacies of either arachidonic acid or β,β′-tetramethylhexadecanedioic acid resulted in only 1–2.5-fold inhibition in the inductive efficacy of 2-methyl-2-[*p*(1,2,3,4-tetrahydro-1-naphthyl)-phenoxy]-propionic acid (nafenopin, Fig. 1D) or

Table 1. Inhibition of PPAR-mediated induction of *AOX PPRE-CAT* by transfected acyl-CoA synthase as a function of CoA thioesterification capacity with the respective peroxisome proliferator. Transfection conditions were as described in the Materials and Methods section and the legend of Fig 1. K_m values for the respective peroxisome proliferator in the absence (−) and presence (+) of transfected acyl-CoA synthase were derived from the fitted curves of Fig 1A–E. The relative catalytic capacity is expressed as the maximal CoA thioesterification velocity for the respective peroxisome proliferator relative to the velocity with palmitate using rat liver microsomal long-chain-fatty-acyl-CoA synthase. The relative maximal velocities for arachidonic acid and nafenopin have been previously reported in [29] and [11], respectively. MEDICA 16, β,β' tetramethylhexadecanedioic acid.

Peroxisomal proliferator	relative V_{max} values for the catalytic capacity of acyl-CoA synthase with peroxisomal proliferators as substrates	inductive efficacy of peroxisomal proliferator		
		K_m for		+ acyl-CoA synthase/ − acyl-CoA synthase
		− acyl-CoA synthase	+ acyl CoA synthase	
		μM		
Arachidonic acid	0.18	11.0	82.5	7.5
MEDICA 16	0.18	50.0	302.0	6.1
Wy 16,463	–	0.15	0.66	4.4
Nafenopin	0.01	11.0	27.5	2.5
Bezafibrate	<0.005	100.0	100.0	1.0

2-[4-(2-[4-chlorobenzamido]-ethyl)phenoxy]-2 methyl-propanoic acid (bezafibrate; Fig. 1E).

The K_m values for the respective peroxisome proliferators in cells transfected and non-transfected with the acyl-CoA synthase are summarized in Table 1 together with the relative V_{max} values for the respective ligands as substrates for acyl-CoA formation catalyzed *in vitro* by rat liver microsomal long chain-acyl-CoA synthase. The inhibitory effect of the transfected acyl-CoA synthase on the inductive efficacy of peroxisome proliferators was negatively correlated with the extent of CoA thioesterification of the respective proliferator (Table 1).

The results presented in this study may indicate that transcriptional *trans*-activation of *AOX* induced by peroxisome proliferators is not mediated by the respective peroxisome-proliferator-CoA. This conclusion is corroborated by the absence of a correlation between the intracellular concentrations of some aryloxy peroxisome-proliferator-CoAs and the pharmacological potency of the respective proliferator precursors [25]. Furthermore, by the same line of evidence, other putative metabolites of xenobiotic peroxisome proliferators which may be derived via the peroxisome-proliferator-CoA intermediates (e.g. carnitine or glycine esters, mixed glycerolipids, acylated proteins) may be similarly ruled out as immediate inducers of peroxisomal *AOX*, thus indicating that the immediate peroxisome-proliferator inducer is rather the free acid, or a derivative thereof, which may be derived from the free acid upon bypassing the CoA thioesterification option. Considering the peroxisomal *AOX* as a representative of genes induced by PPAR raises the possibility that this conclusion may apply to other genes induced by peroxisome proliferators.

The activity of the free acid is in agreement with previous studies concerning the induction of peroxisomal activities by perfluorocarboxylic or perfluorosulphonic acids [26, 27]. The perfluoro compounds are essentially non-metabolic and do not share any metabolic pathway with amphipathic carboxylates acting as peroxisome proliferators. All of these molecules do however share the common characteristic of having an acidic moiety attached to a hydrophobic backbone. Hence, the free acidic group is presumably directly involved in initiating the inductive process while the nature of the hydrophobic backbone determines the overall inductive efficacy. It still has to be investigated whether induction is indeed initiated by binding of the peroxisome proliferator free acid to PPAR (albeit with low binding affinity [4]) as suggested by [28] in view of the relationship observed between the respective inductive efficacies of some proliferators and their binding affinities to PPAR deduced by molecular modelling. Alternatively, peroxisome proliferators could indirectly affect the affinity of PPAR for PPRE by modulating the PPAR phosphorylation/oxidation state rather than by directly binding to the putative ligand-binding site. The extreme structural diversity of peroxisome proliferators is indeed in contrast with the strict structural specificity required for ligand binding by various members of the superfamily receptors.

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